Gene Expression FFPE Workflow

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What is Sigma WTA

The TransPlex Whole Transcriptome Amplification (WTA) kit allows for rapid amplification of total RNA from formalin-fixed, paraffin embedded (FFPE) samples in less than 4 hours without 3'-bias. The WTA kit involves two steps. First, total RNA is reverse transcribed with a WTA Polymerase using non-complementary primers composed of quasi-random 3' end and a universal 5' end. Then, the resulting Omniplex cDNA library, composed of overlapping 100 to 1000 base fragments, is PCR amplified to produce microgram quantities of WTA products for downstream applications such as qPCR and microarray analyses.

NOTE Sigma makes two different WTA kits: WTA1 and WTA2. This protocol uses the WTA1 kit.

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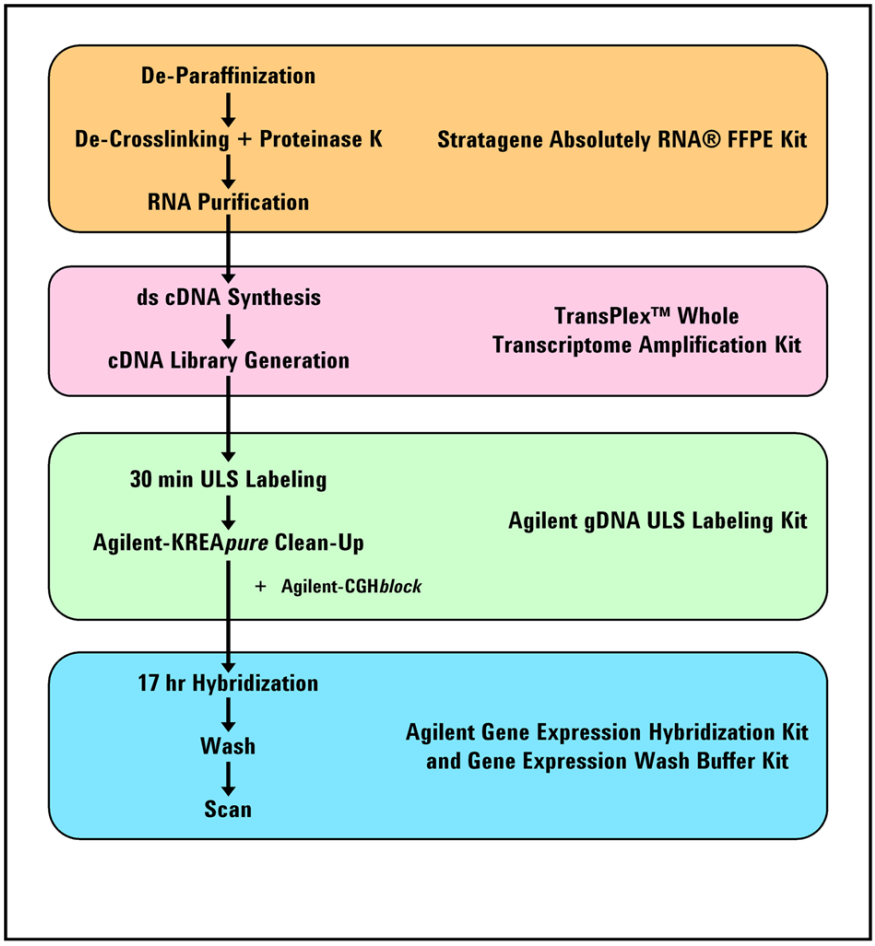

Figure 1 Various kits and steps involved in the analysis of FFPE samples with the Agilent gene expression microarray workflow.

Before you Begin

Make sure you have the material listed in this section.

Required Equipment

Table 1 Required Equipment

Description	Vendor and part number
Agilent Microarray Scanner	Agilent p/n G2565CA or G2565BA
Hybridization Chamber, stainless	Agilent p/n G2534A
Hybridization Chamber gasket slides	
1 microarray/slide, 5 slides/box	Agilent p/n G2534-60003
2 microarrays/slide, 5 slides/box	Agilent p/n G2534-60002
4 microarrays/slide, 5 slides/box	Agilent p/n G2534-60011
8 microarrays/slide, 5 slides/box	Agilent p/n G2534-60014
Hybridization oven; temperature set at 65°C	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
Nuclease-free 1.5 mL microfuge tubes	Ambion p/n 12400 or equivalent
Magnetic stir bar (×2)	Corning p/n 401435 or equivalent
Magnetic stir plate (×2)	Corning p/n 6795-410 or equivalent
Microcentrifuge	Eppendorf p/n 5417R or equivalent
NanoDrop ND-1000 UV-VIS spectrophotometer	NanoDrop p/n ND-1000 or equivalent
Slide-staining dish, with slide rack (×3)	Thermo Shandon p/n 121 or equivalent
Thermocycler with heated lid	
96-well PCR plate or PCR tubes	
Clean forceps	
Ice bucket	
Pipetman micropipettors, (P-10, P-20, P-200, P-1000) or equivalent	
Powder-free gloves	

Table 1 Required Equipment (continued)

Description	Vendor and part number
Sterile, nuclease-free aerosol barrier pipette tips	
Vortex mixer	
Timer	
Nitrogen purge box for slide storage	

Required Reagents

Table 2 Required Reagents

Description	Vendor and part number
Genomic DNA ULS Labeling Kit	Agilent p/n 5190-0419
Gene Expression Hybridization Kit	Agilent p/n 5188-5242
Gene Expression Wash Buffer 1	Agilent p/n 5188-5325
Gene Expression Wash Buffer 2	Agilent p/n 5188-5326
100% Ethanol	Amresco p/n E193
TITANIUM Taq DNA Polymerase	Clontech p/n 639208
DNase/RNase-free distilled water	Invitrogen p/n 10977-015
QIAquick PCR Purification	Qiagen p/n 28106
Sulfolane	Sigma p/n T22209
Transplex Whole Transcriptome Amplification kit	Sigma p/n WTA1-50RXN
Absolutely RNA FFPE Kit	Stratagene p/n 400809
Milli-Q water or equivalent	

Optional Equipment/Reagents

Table 3 Optional Equipment/Reagents

Description	Vendor and part number
2100 Bioanalyzer	Agilent p/n G2938A
RNA 6000 Nano Assay Kit (RNA Series II Kit)	Agilent p/n 5067-1511
DNA 1000 Kit	Agilent p/n 5067-1505
Stabilization and Drying Solution	Agilent p/n 5185-5979
Slide box	Corning p/n 07201629
Acetonitrile	Sigma p/n 271004-1L

Procedure

Step 1. Isolate RNA

1 Extract RNA from FFPE blocks. Follow the instructions in the user manual for the Stratagene Absolutely RNA FFPE Kit (cat # 400809) at http://www.stratagene.com/manuals/400809.pdf.

For the Proteinase K Digestion step of the Stratagene Absolutely RNA FFPE manual, incubate the tubes at 55°C for 18 hours instead of 3 hours.

Step 2. Prepare WTA Library

1. Thaw the WTA Library Synthesis Buffer and WTA Library Stabilization on ice and mix on a vortex mixer. If either solution has a precipitate, briefly heat at 37°C and mix the tube(s) on a vortex mixer until the precipitate is gone.
2. Add nuclease-free water to 25 to 300 ng of FFPE-extracted RNA to get a total volume of 19 µL (use tubes/strips/plates that will fit in a PCR thermal cycler).
3. Prepare the following library preparation mix:

Table 4 WTA Library Preparation Mix

Components	Per reaction (µL)	Per 4 reactions (µL) (including excess)
WTA Library Synthesis Buffer	2.5	12.5
WTA Library Stabilization Solution	2.5	12.5
Final Volume of Library Prep Master Mix	5	25

4. Add 5 µL of the Library Preparation Mix (Table 4) to FFPE-extracted RNA for a total volume of 24 µL.
5. Mix samples well by pipetting and incubate at 70°C for 5 minutes.
6. Cool reaction on ice and briefly centrifuge liquid to bottom of PCR plate.
7. Add 1 µL of Library Synthesis Enzyme to each sample for a total volume of 25 µL and mix well by pipetting.
8. Place PCR plate in thermal cycler and incubate as follows:
   - 24°C for 15 minutes
   - 42°C for 2 hours
   - 95°C for 5 minutes
   - 4°C - hold
9. Cool reaction on ice and briefly centrifuge PCR plate.

Step 3. Amplify WTA Library

1 Immediately prior to use, gently mix the components listed in Table 5 for the WTA Amplification Mix by adding in the order indicated, and keep on ice.

Clontech Titanium Taq DNA Polymerase is an enzyme, which needs to be kept on ice and added to the WTA Amplification Mix just before starting the reactions.

NOTE The Titanium Taq DNA Polymerase is purchased separately from Clontech.

Table 5 WTA Amplification Mix

Components	Per reaction (µL)	Per 4 reactions (µL) (including excess)
Nuclease-free water	300	1500
WTA Amplification Master Mix	37.5	187.5
dNTP Mix	7.5	37.5
Clontech Titanium Taq DNA Pol	5	25
Final Volume of Amp Master Mix	350	1750

2 Divide the library generated in "Step 2. Prepare WTA Library" (25 µL) into 5 equivalent 5 µL aliquots in the wells of a PCR plate.

3 Add 70 µL of the WTA Amplification Mix (Table 5) to each library aliquot and mix well by gently pipetting up and down. The final reaction volume should be 75 µL.

4 Place PCR plate in thermal cycler and cycle as follows:

- 95°C for 3 minutes
- 94°C for 20 seconds, 65°C for 5 minutes, for 22 cycles
- 4°C - Hold

5 Cool reaction on ice and briefly centrifuge PCR plate.

Make sure that the amplified library aliquots are purified and pooled before further analysis. The purified and pooled amplified library can be stored at -20°C.

Step 4. Purify WTA products using the QIAquick PCR Purification Kit

All centrifuge steps are at 13,000 rpm (approximately 17,900 x g) in a conventional tabletop microcentrifuge.

NOTE The QIAquick kit is purchased separately from Qiagen.

NOTE For best results, purify individual amplified aliquots separately, then pool them together.

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix (for example, 375 µL Buffer PB to 75 µL PCR product).
2. Put a QIAquick spin column in provided 2 mL collection tube.
3. To bind DNA to the column, apply the sample to the QIAquick column and spin in a centrifuge for 30 to 60 seconds.
4. Discard the flow-through. Place the QIAquick column back into the same tube.
5. To wash, add 0.75 mL Buffer PE to the QIAquick column and centrifuge for 30 to 60 seconds.
6. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 minute.
7. Place QIAquick column in a clean 1.5 mL microcentrifuge tube.
8. To elute the DNA, add 50 µL nuclease-free water to the center of the QIAquick membrane, let the column stand for 1 minute, and then spin the column in a centrifuge for 1 minute.
9. Move to ice.
10. Quantitate with NanoDrop using 1.5 µL of the eluted DNA.

Step 5. Label with ULS

1 For a 4-pack microarray hybridization, prepare a tube that contains 1.65 µg of cDNA in a final volume of 16.35 µL.

If the 1.65 µg of cDNA is in a volume greater than 16.35 µL, use a vacuum concentrator to concentrate the sample until the volume is 16.35 µL.

1 Mix the components in Table 6 on ice to prepare one Cy3 labeling master mix:

Table 6 Preparation of Labeling Master Mix (for 4-pack using FFPE samples)

Components	Per reaction (µL)	Per 4 reactions (µL) (including excess)
ULS-Cy3	1.65	8.25
10x Labeling Solution	2	10
Final Volume of Labeling Master Mix	3.65	18.25

2 Add 3.65 µL of Labeling Master Mix to each tube that contains the cDNA for a total volume of 20 µL. Mix well by gently pipetting up and down.

3 Incubate tubes at 85°C for 30 minutes.

4 Transfer samples to ice and incubate on ice for at least 3 minutes.

5 Spin in a microcentrifuge for 1 minute at 6000 x g to drive contents off the walls and lid.

Store labeled cDNA on ice until excess dye is removed using the Agilent KREApure columns.

Step 6. Remove non-reacted Cy-ULS

1. Resuspend Agilent KREApure column material by briefly mixing on a vortex mixer.
2. Loosen cap ¼ turn and snap off the bottom closure.
3. Put the Agilent KREApure column in a microcentrifuge and spin for 1 minute at maximum speed (minimum 16000 x g).
4. Discard the cap and flow-through, and place the Agilent KREApure column back into the same collection tube.
5. Add 300 µL water to the column.
6. Spin for 1 minute at maximum speed (minimum 16,000 x g).
7. Discard the flow-through and collection tube.
8. Transfer the column to a clean 1.5 mL microcentrifuge tube.
9. Add the ULS-labeled sample from "Step 5. Label with ULS" (20 µL for 4-pack) to the center of the column membrane.
10. Spin for 1 minute at maximum speed (minimum 16,000 x g) to collect the purified labeled sample in the collection tube.
11. Quantitate the labeled cDNA with a NanoDrop using 1.5 µL of the eluted sample. Make sure 18.5 µL of the ULS-labeled sample remains in each tube.

$$\text{Degree of labeling} = \frac{340 \times \text{pmol per µL dye}}{\text{ng per µL cDNA} \times 1000} \times 100\%$$

The optimal Degree of labeling is between 1.5% and 3.0%

NOTE Because ULS labeling does not copy or amplify, the yield after labeling will be the same as the input amount of cDNA.

Step 7. Prepare labeled cDNA for hybridization

1 Prepare 100X Blocking Agent:

   a Add nuclease-free water to the vial of lyophilized 10X GE Blocking Agent, depending on the Gene Expression Hyb Kit used:

Table 7 Nuclease-free water volume

Components	Nuclease-free Water (µL)*
For Agilent Gene Expression Hyb Kit	50
For Hi-RPM Gene Express Hyb Kit	125

* These volumes are 10% of the amount instructed with the Hyb Kit.

   b Mix briefly on a vortex mixer and leave at room temperature for 60 minutes to reconstitute sample before use or storage.

   c Cross out 10X on the label on the blocking agent vial and write 100X.

2 Bring the Agilent-CGHblock (supplied with the ULS Labeling Kit) to room temperature, for use in step 9.

NOTE Agilent-CGHBlock contains components that cannot be heated to 95°C.

3 Equilibrate water baths or heat blocks to 95°C.

4 Mix the components below to prepare the Hyb Master Mix:

Table 8 Preparation of Hybridization Master Mix

Components	Per reaction (µL)	Per 4 reactions (µL) (including excess)
Nuclease-free water	8.4	42
100X GE Blocking Agent	1.1	5.5
2x Hi-RPM GE Hyb Buffer	55	275
Final Volume of Hyb Master Mix	64.5	322.5

5 Add 64.5 µL of the Hyb Master Mix to the 18.5 µL of ULS-labeled cDNA (from step 11 on page 12) for a total volume of 83 µL.
6 Incubate at 95°C for 3 minutes, then place on ice.
7 Spin samples briefly in a microcentrifuge to drive contents off the walls and lid.
8 Make sure that the Agilent-CGHblock is completely equilibrated to room temperature before you continue.
9 Add 27 µL of Agilent-CGHblock to each sample for a final volume of 110 µL, and store at room temperature.

   Make sure the samples are kept at room temperature until you dispense them onto the arrays.

NOTE The addition of Agilent-CGHBlock to the hybridization is needed to eliminate background noise on the microarray.

10 Dispense 100 µL of sample onto each 4x44K microarray.
11 Hybridize at 65°C for 17 hours at 20 RPM.
12 Use standard Agilent Gene Expression wash and scan conditions.

   For details, refer to the *One-color Microarray-based Gene Expression Analysis (Low Input Quick Amp Labeling) Protocol*, p/n G4140-90040.

Microarray QC Metrics for FFPE samples

These metrics are only appropriate for samples analyzed with Agilent Gene Expression microarrays by following the standard operational procedures provided in this FFPE Quick Start Guide. These metrics are exported to a table in the Feature Extraction QC report. The QC metrics can be used to assess the relative data quality across a set of microarrays in an experiment. In some cases, they can indicate potential processing errors that may have occurred or suggest that the data from particular microarrays might be compromised. Many factors can influence the range of these metrics including the biological sample source, quality of starting FFPE samples, experimental processing, scanner sensitivity and image processing. The value guidelines presented below represent the thresholds that Agilent has observed when analyzing FFPE samples using this protocol.

Table 9 QC metric thresholds for labeling

	FFPE Samples
AnyColorPrcntFeatNonUnifOL	< 1
gNegCtrlAveNetSig	< 100
gNegCtrlAveBGSubSig	-10 to 5
gNegCtrlSDevBGSubSig	< 10
gSpatialDetrendRMSFit	< 15
gNonCntrlMedCVProcSig	0 to 8

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In This Book

The *Quick Start Guide* presents overview instructions to process FFPE RNA samples.

These instructions are based on the “Gene Expression Microarray Analysis of Archival FFPE Samples” Application Note, p/n 5990-3917EN.

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